

Expression of Cholesterol Sulfotransferase (SULT2B1b) in Human Platelets

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Background—Cholesterol sulfate, the most important sterol sulfate in the human circulation, has emerged as a multifaceted molecule. Among its many demonstrated regulatory actions is its ability to influence blood clotting and fibrinolysis. Additionally, cholesterol sulfate is a constituent of human platelets, where it has been shown to support platelet aggregation.

Methods and Results—We have documented the presence of the enzyme (SULT2B1b) that sulfonates cholesterol in human platelets and examined the influence of plasma lipoproteins on the expression and activity of this enzyme. SULT2B1b mRNA was detected by reverse transcription–polymerase chain reaction and found to be the only steroid/sterol sulfotransferase expressed in these discoid nucleate particles. Using real-time polymerase chain reaction for quantification, we found that the level of SULT2B1b mRNA in platelets was maintained at 4°C but substantially diminished over a period of 4 hours at 37°C. The loss of SULT2B1b mRNA, however, was markedly reduced in the presence of HDL but not LDL. The stabilizing influence of HDL was attributable specifically to its apolipoprotein (apo) A-I component, whereas apoA-II and apoE were without effect. Importantly, there was a direct correlation between platelet SULT2B1b mRNA and protein levels in the presence or absence of lipoprotein that was reflected in enzymatic activity and cholesterol sulfate production.

Conclusions—Human platelets selectively express SULT2B1b, the physiological cholesterol sulfotransferase. Furthermore, the stability of SULT2B1b mRNA and protein in platelets maintained at 37°C is subject to regulation by the apoA-I component of HDL. (*Circulation*. 2004;109:92-96.)

Key Words: platelets ■ lipoproteins ■ apolipoproteins ■ cholesterol

Cholesterol sulfate is quantitatively the most important known sterol sulfate in the human circulation, where its concentration ranges from 2 to 6 $\mu\text{mol/L}$ ¹⁻⁵; furthermore, it is widely distributed in human tissues^{6,7} and is a normal constituent of red blood cells^{1,4,8-10} and platelets.^{11,12} Interestingly, cholesterol sulfate has emerged as a multifaceted regulatory molecule.¹³ Its presence in cell membranes protects erythrocytes against osmotic lysis^{10,14} and regulates sperm capacitation.^{15,16} In platelets, cholesterol sulfate has been shown to support platelet adhesion.^{11,17}

Although the content of cholesterol sulfate in human platelets has been estimated,¹¹ the source of this sulfolipid has not been determined. That is, the cholesterol sulfate that is present in platelets could be derived from the circulation or could arise from endogenous platelet production. The enzyme that catalyzes the sulfoconjugation of cholesterol is part of a superfamily of cytosolic sulfotransferases (SULTs) that catalyze the sulfoconjugation of hormones and neurotransmitters as well as drugs and xenobiotics.¹⁸ The SULT superfamily is composed of 5 families, 1 of which (SULT2) is primarily engaged in the sulfoconjugation

of neutral steroids and sterols.¹⁹ The SULT2 family is divided into 2 subfamilies, SULT2A1 and SULT2B1, and the SULT2B1 subfamily is additionally divided into 2 isoforms, SULT2B1a and SULT2B1b.²⁰ Importantly, SULT2B1b functions as a selective cholesterol sulfotransferase.²¹ Thus, we have analyzed human platelets for expression of SULT2B1b as well as the expression of the other SULT2 isozymes, which have detectable but weak cholesterol sulfotransferase activity.²¹ Furthermore, based on the known interaction of plasma lipoproteins with platelets,²² we explored the influence of lipoproteins on SULT2B1b mRNA and protein expression as well as cholesterol sulfotransferase activity and cholesterol sulfate production.

Methods

Materials

Cholesterol (purity >99%, standard for chromatography), cholesterol sulfate, coprostanol (purity >98%), methylene blue, LDL (aseptically filtered), HDL (aseptically filtered), and 3'-phosphoadenosine 5'-phosphosulfate (PAPS; purity >80% by high-performance liquid chromatography [HPLC]) were purchased from Sigma. Apolipopro-

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tein (apo) A-I (purity >95%), apoA-II (purity >95%), and apoE (purity >95%) were obtained from Calbiochem. EDTA solution (certified DNase/RNase-free) and Dulbecco's Ca^{2+} - and Mg^{2+} -free PBS (DNase/RNase-free) were obtained from Research Genetics and Mediatech, respectively. Methanol (100%, for use in liquid chromatography and spectrometry), chloroform (100%), and ethanol (100%) were purchased from Mallinckrodt Baker. Oligonucleotides were obtained from Gene Probe Technologies. Absolutely RNA reverse transcription-polymerase reaction (RT-PCR) Miniprep Kit and XL-1 blue were purchased from Stratagene. ThermoScript RT-PCR system, Platinum Taq DNA polymerase, pCR2.1 vector, and TOPO TA cloning kit were purchased from Invitrogen. QIAprep Spin Minikit was purchased from Qiagen. Skin cDNA was produced using skin RNA from Clontech.

Platelet Preparation

Platelets were prepared from blood obtained from overnight-fasted human volunteers. All plastic tubes used throughout this study were composed of polypropylene. Briefly, platelet-rich plasma was prepared by centrifuging blood anticoagulated with EDTA at 250g for 10 minutes at 4°C. To prepare a washed platelet suspension (WPS), platelet-rich plasma was diluted with an equal volume of PBS (Mediatech) and centrifuged at 650g for 10 minutes at 4°C. The platelet pellet was resuspended in PBS and centrifuged at 650g for 10 minutes at 4°C; this step was repeated a second time. The final pellet was suspended in 1.0 mL of PBS to achieve a final concentration of 4.5×10^9 platelets/mL.

RT-PCR Analysis of SULT2A1, SULT2B1a, and SULT2B1b Expression by Platelets

Total RNA was extracted from washed platelets using Absolutely RNA RT-PCR Miniprep Kit according to the manufacturer's instructions (Stratagene). RNA was placed in a low-salt wash buffer and digested with DNase to remove any remaining DNA. RT was performed using the ThermoScript RT-PCR system according to the manufacturer's instructions (Invitrogen). Briefly, using 0.5 μg of total RNA as a template, first-strand cDNA was made using 25 pmol of oligo(dT)₂₀ and 25 ng of random hexamer primer (Invitrogen) in a 20- μL reaction volume. After heat denaturing at 65°C for 5 minutes, RT was carried out at 25°C for 10 minutes and then 60°C for 50 minutes. A 2- μL aliquot of cDNA was used as template. Primers used were 5'-C_(nt295)ACCTCC-CCATCCAGTATTCC_(nt1316)-3' (sense) and 5'-G_(nt581)TTCTTCC-TGTGTCCTGTTTCAGC_(nt5581)-3' (antisense) for SULT2A1, 5'-T_(nt269)CACCACTTTACAGAAGA-GGGACTG_(nt293)-3' (sense) and 5'-G_(nt567)ATCTCGATCATCCAGGTCGTG_(nt546)-3' (antisense) for SULT2B1a, 5'-G_(nt112)GCTGTGGGACACCTATGAAG_(nt133)-3' (sense) and 5'-A_(nt317)TCTCGATCATCCAGGTCGTG_(nt296)-3' (antisense) for SULT2B1b, and 5'-C_(nt261)TGGCACCACA-CCTTCTAC-AATG_(nt283)-3' (sense) and 5'-A_(nt651)ATGTCACGCACGATTCC-CGC_(nt630)-3' (antisense) for β -actin. Expected sizes of SULT2A1, SULT2B1a, SULT2B1b, and β -actin were, respectively, 286, 299, 206, and 369 bp. PCR conditions were as follows: denaturing at 95°C for 5 minutes, followed by 30 cycles of denaturing at 94°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 30 seconds. PCR products were analyzed by electrophoresis using 3% agarose gels.

Real-Time RT-PCR Analysis of Platelet SULT2B1b Expression and the Effect of Lipoproteins and Apolipoproteins

WPS (0.5 mL of 4.5×10^9 platelets/mL) was incubated with and without LDL or HDL (100 $\mu\text{g}/\text{mL}$, final concentration) for 0, 15, 30, 60, 120, and 240 minutes at 37°C; incubations were also carried out at 4°C for 0, 120, and 240 minutes. In other experiments, WPS was incubated with and without apoA-I, apoA-II, or apoE (100 $\mu\text{g}/\text{mL}$, final concentration) for 0, 120, and 240 minutes at 37°C. SULT2B1b mRNA was quantified by real-time RT-PCR performed using a fluorescence temperature cycler (LightCycler) and SYBR Green I as a double-strand DNA-specific binding dye according to the manufacturer's instructions (Roche Molecular Biochemicals) using the same primers that were used with RT-PCR. This technique continuously

monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. Amplifications were carried out using 1 U Platinum Taq DNA polymerase (Invitrogen), 0.5 $\mu\text{mol}/\text{L}$ of each primer, 3 $\mu\text{mol}/\text{L}$ MgCl_2 , 10 \times Platinum Taq DNA polymerase buffer (200 $\mu\text{mol}/\text{L}$ Tris-HCl, pH 8.4, 500 $\mu\text{mol}/\text{L}$ KCl), 0.2 $\mu\text{mol}/\text{L}$ dNTP, 1 mg/mL BSA, 1 μL 1:2000 dilution of SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications), and 2 μL 1:5 dilution of cDNA in a total volume of 20 μL . The real-time PCR conditions were preheat denaturation at 95°C for 5 minutes, annealing 59°C for 10 seconds, and extension 72°C for 7 seconds; cycle number 45. SYBR Green I fluorescence was detected at 72°C at the end of each cycle to monitor the amount of PCR product formed during that cycle. A melting curve analysis of the amplification products was performed at the end of the PCR run by rapidly increasing the temperature to 95°C, followed by immediate cooling to 65°C for 15 seconds, after which the temperature was gradually increased to 95°C at a rate of 0.1°C per second, with continuous measurement of fluorescence to confirm amplification of specific transcripts. The melting temperature profile for SULT2B1b and β -actin demonstrated single peaks at 89°C and 88°C, respectively. The interassay and intraassay coefficients of variation were calculated to be 7.4% and 5.7%, respectively, using SULT2B1b primer and skin cDNA. External cDNA standards for SULT2B1b and β -actin were produced by inserting PCR products, which were generated using the same primers used for RT-PCR and skin cDNA as a template, into the pCR2.1 vector using the TOPO TA Cloning Kit (Invitrogen). Vector constructs were used to transform XL1-blue (Stratagene), and plasmid DNA was prepared by QIAprep Spin Miniprep Kit (Qiagen). The inserts of control vector for SULT2B1b and β -actin were verified by sequencing. The concentration of standard was determined by measuring the OD_{260} , and the copy number was calculated.

Western Blot Analysis

Washed platelets were suspended in 0.5 mL PBS, sonicated for 15 seconds twice on ice, and concentrated using Microcon (Millipore). Protein concentrations were determined using the BCA protein assay kit (Pierce). Platelet extracts (70 μg) as well as an extract of human skin as a positive control (30 μg) were electrophoresed on a 10% Bis-Tris gel and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were soaked in a solution of 5% dry milk (Bio-Rad) in TBS containing 0.05% Tween 20 for 30 minutes with gentle shaking, after which they were exposed to SULT2B antibody (1:40) for 3 hours. Membranes were washed 3 times in TBS containing 0.05% Tween 20 and incubated with goat anti-rabbit antibody (1:55 000, KPL) for 30 minutes. Finally, membranes were washed 3 times in TBS containing 0.05% Tween 20, and signals were detected using LumiGLO (KPL) according to the manufacturer's protocol before exposure to Scientific Imaging Film (Kodak).

Determination of Cholesterol Sulfate Content in Platelets by HPLC

WPS (1.0 mL of 4.5×10^9 platelets/mL) was incubated with and without LDL or HDL (100 $\mu\text{g}/\text{mL}$, final concentration) in addition to 4.5×10^{-6} mol/L PAPS for 240 minutes at 37°C. To extract and measure cholesterol sulfate, the method using methylene blue and saturated Na_2SO_4 in H_2SO_4 was used.²³ Briefly, after incubation, platelets were washed twice, resuspended in 0.5 mL PBS, and sonicated for 15 seconds twice on ice. Ethanol (4.5 mL) was added, and samples were placed on ice for 20 minutes and then centrifuged at 16 000g for 10 minutes at 4°C. The decanted supernatant was evaporated under nitrogen at 40°C. Chloroform (1 mL) was added along with 100 μL of methylene blue reagent (0.78 mmol/L methylene blue, 0.35 mol/L Na_2SO_4 , and 1/100 [vol/vol] H_2SO_4 in water) and 4 mL water. The aqueous phase was discarded. The addition of water was repeated until the aqueous phase was transparent and colorless. The chloroform phase, which was blue colored, was collected and evaporated under nitrogen at 40°C, and the residue was taken up in 125 μL of methanol. A linear standard curve of methylene blue/sulfated sterol complex (OD_{660}) was constructed with 2.19, 4.38, 6.57, 8.76, and 10.95 pmol cholesterol sulfate, which had a correlation coefficient of $r=0.999$ ($P<0.0001$, Fisher's Z transformation).

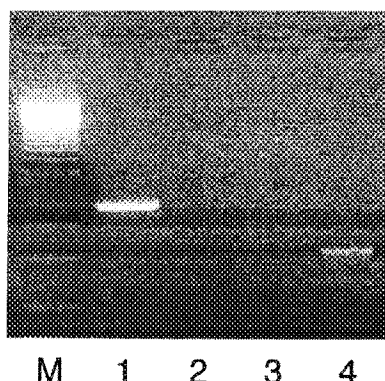


Figure 1. RT-PCR revealed that only SULT2B1b was expressed in human platelets, as indicated in lane 4, whereas SULT2A1 (lane 2) and SULT2B1a (lane 3) were not expressed; β -actin expression is denoted in lane 1. M indicates molecular markers.

Cholesterol sulfate was assayed by HPLC based on a previously described procedure.²⁴ Briefly, model No. 1100 HPLC instrument (Hewlett-Packard), equipped with a diode array detector set to absorb at 202 ± 20 nm, was used. A linear standard curve was developed with 4.88, 9.77, 19.4, and 38.8 μ g of cholesterol sulfate that had correlation coefficient of $r=0.989$, $P<0.0002$ (Fisher's Z transformation) between the mass of cholesterol sulfate and the area under the curve of the cholesterol sulfate peak. Fifty microliters of each sample were injected onto an Aqua 3 C18 reverse-phase column, 150×4.6 cm (Phenomenex). An initial solvent system of methanol and water (60:40), each containing 1.0 mL of 7.4 mol/L ammonium acetate per liter, was used. During a 40-minute period, the solvent system changed linearly to 100:0 at the speed of 0.6 mL/min; this was followed by an additional isocratic period of 20 minutes, for a total duration of 60 minutes. Fractions were collected at intervals of 3.0 minutes.

Mass spectrometry was used to confirm the identity of the sterol moiety of the fraction collected by HPLC that was identical in retention time to authentic cholesterol sulfate. Coprostanol was used as an internal standard. Samples were solvolyzed, extracted into chloroform, and derivatized with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (Pierce). The *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide ethers were injected in the split mode onto a capillary column (ZB 1701, Phenomenex) with an initial temperature of 240°C and temperature programming at $1.5^\circ\text{C}/\text{min}$ for 30 minutes. The SCAN and SIM modes were used for analysis. In the SIM mode, m/z 368 and 370 were used for the detection of cholesterol and coprostanol, respectively.

Results

Effect of Lipoproteins and Apolipoproteins on SULT2B1b mRNA Stability

SULT2B1b mRNA is clearly expressed in human platelets, whereas the other SULT2 isozymes, ie, SULT2A1 and SULT2B1a, are not expressed by these anucleate particles (Figure 1). When platelets were incubated in PBS at 37°C , the amount of SULT2B1b mRNA was reduced to 40% of the quantity of the starting material over the first 30 minutes; the rate of decay then slowed, and by 4 hours, 20% remained (Figure 2). In the presence of LDL, the rate of disappearance of SULT2B1b mRNA at 37°C was even more rapid over the first 30 minutes, and after 4 hours only 10% remained. In the presence of HDL, however, the decay of platelet SULT2B1b mRNA at 37°C was significantly reduced, and $\approx 50\%$ remained after 4 hours (Figure 2). On the other hand, at 4°C in

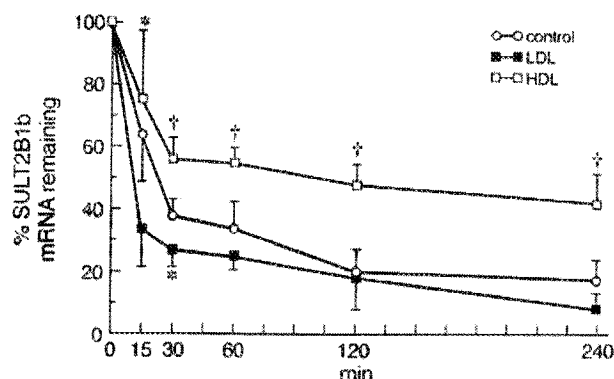


Figure 2. HDL (\square) but not LDL (\blacksquare) significantly retarded the in vitro decay of SULT2B1b mRNA when human platelets were incubated at 37°C in PBS for 4 hours. \circ , Control values. Data points represent mean \pm SD ($n=4$). $*P<0.05$; $\dagger P<0.005$ vs controls using Scheffé's *F* test.

PBS, SULT2B1b mRNA is much more stable, and there was no significant difference in the quantity of SULT2B1b mRNA in the presence or absence of either LDL or HDL over the 4-hour period (Figure 3). During these studies, the expression pattern of β -actin mRNA did not vary substantially at either temperature used (data not presented).

We next studied the effect of individual apolipoprotein constituents of HDL on SULT2B1b mRNA stability at 37°C in PBS. The quantity of SULT2B1b mRNA varied little during the 4-hour period when platelets were incubated with apoA-I (Figure 4). On the other hand, the amount of SULT2B1b mRNA in platelets incubated at 37°C in PBS with either apoA-II or apoE was reduced by $\approx 70\%$ (Figure 5). Again, there was no significant difference in β -actin mRNA expression in these platelet preparations (data not presented).

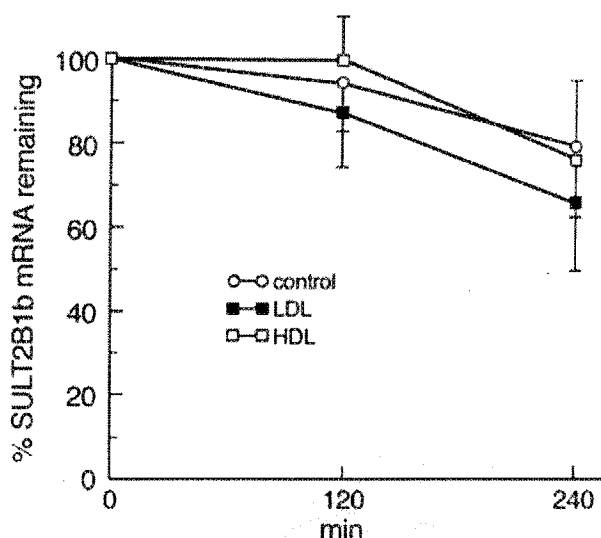


Figure 3. In contrast to the degradation of SULT2B1b mRNA that occurred at 37°C , SULT2B1b mRNA at 4°C was fairly stable, and at this temperature there was no significant difference between the content of SULT2B1b mRNA in the presence and absence of lipoproteins (compare with Figure 2).

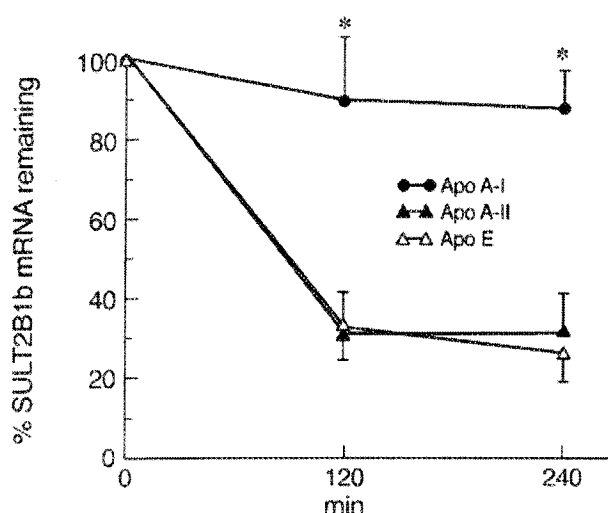


Figure 4. Stabilizing effect of HDL on SULT2B1b mRNA at 37°C was attributable specifically to the apoA-I constituent of HDL, whereas apoA-II and apoE were without effect. Data points indicate mean \pm SD ($n=4$). * $P<0.0001$ vs apoA-II and apoE using Scheffé's F test.

Effect of Lipoproteins on Expression of the SULT2B1b Protein

Western blot analysis (Figure 5) revealed that the level of SULT2B1b protein essentially paralleled the changes in the SULT2B1b mRNA levels shown in Figure 2. That is, the SULT2B1b protein was reduced by approximately one half after maintaining platelets at 37°C for 4 hours in the presence of HDL (Figure 5). In contrast, no SULT2B1b protein was detectable after 4 hours at 37°C in the presence of LDL or in the absence of any lipoprotein (Figure 5).

Effect of Lipoproteins on Cholesterol Sulfate Synthesis in Platelets

The peak appearance and retention time of cholesterol sulfate were highly reproducible after HPLC; furthermore, the identity of cholesterol sulfate in the HPLC cholesterol sulfate fraction was confirmed by staining with methylene blue and gas liquid chromatography mass spectrometry (data not presented). The retention time of cholesterol sulfate and cholesterol was 32.63 ± 0.33 and 45.92 ± 0.001 minutes, respectively (mean \pm SD). Notably, the narrow retention peaks suggested that contaminants were unlikely to be present.

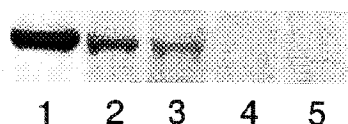


Figure 5. Expression of SULT2B1b protein by human platelets in the presence and absence of lipoproteins. Western blot analysis demonstrated that SULT2B1b was present in preincubated platelets (lane 2) as well as platelets incubated for 240 minutes at 37°C in the presence of HDL (lane 3), whereas SULT2B1b was not detectable in platelets incubated for 240 minutes at 37°C in the presence of LDL (lane 4) or no lipoprotein addition (lane 5). Human skin tissue was used as a positive control for SULT2B1b expression (lane 1).

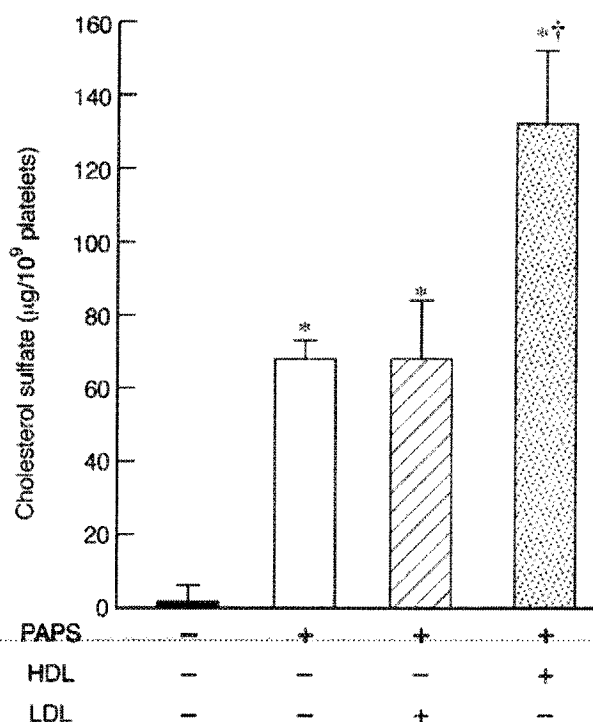


Figure 6. Cholesterol sulfate content in human platelets incubated at 37°C for 240 minutes with and without PAPS and with and without HDL or LDL. The top of each column represents the mean value ($n=4$), and error bars indicate SD. * $P<0.005$ vs platelets incubated without PAPS; † $P<0.005$ vs platelets incubated with either PAPS and LDL or PAPS and no lipoprotein using Scheffé's F test.

The cholesterol sulfate content in control platelets was 566 ± 62 pmol/ 10^9 platelets. The addition of PAPS to control platelet preparations dramatically increased the cholesterol sulfate content to levels of ≈ 150 $\mu\text{g}/10^9$ platelets (Figure 6). Whereas cholesterol sulfate production was not additionally increased in the presence of LDL, the amount of cholesterol sulfate produced in the presence of HDL essentially doubled that of the control and LDL samples (Figure 6).

Discussion

Cholesterol sulfate has been shown to be a normal constituent of blood platelets and to modulate platelet function.¹¹ For example, it potentiates ADP- and thrombin-induced platelet aggregation as well as serotonin secretion. These effects are specific for cholesterol sulfate and require both the sterol ring structure as well as the sulfate moiety.¹¹ Furthermore, cholesterol sulfate modulates arachidonic acid metabolism while potentiating arachidonic acid-induced platelet aggregation, effects that are, in part, explained by changes in calcium flux.¹⁷ It has also been shown that platelets will adhere to cholesterol sulfate but not to cholesterol, other cholesterol esters, or other steroid sulfates under flow conditions similar to those seen in arteries.¹²

It is now appreciated that SULT2B1b is the physiological cholesterol sulfotransferase.²¹ SULT2B1b is selectively expressed in specific tissues, where it is known that cholesterol sulfate plays an important physiological role, eg, skin.²⁵ We

now report, for the first time, that SULT2B1b mRNA is selectively expressed in human platelets, whereas mRNAs for the other known SULT2 isozymes, ie, SULT2A1 and SULT2B1a, are not expressed by these discoid anucleate particles. Although platelets lack a nucleus, they do contain a rough endoplasmic reticulum and polysomes and are known to engage in protein synthesis.²⁶ The presence of SULT2B1b protein in platelet preparations was confirmed by Western analysis as well as by measuring catalytic activity and the content of cholesterol sulfate produced. The finding of 566 pmol of cholesterol sulfate per 10^9 untreated platelets is in keeping with a previous report where the content of cholesterol sulfate in human platelets was found to range from 164 to 512 pmol/ 10^9 platelets.¹¹ The remarkable increase in platelet cholesterol sulfate content (≈ 300 -fold) after the addition of exogenous PAPS to the incubation medium was totally unexpected. This clearly suggests that PAPS was limiting in our platelet preparations, although it is not clear why, because these platelets expressed mRNA for PAPS synthase 1 (data not presented), the enzyme that catalyzes the formation of PAPS from ATP and inorganic sulfate.²⁷ It thus seems that platelets have a huge potential for synthesizing cholesterol sulfate, but this activity normally may be kept in check by regulating the availability of the sulfonate donor molecule PAPS. This could have important physiological significance regarding platelet activation and adhesiveness.

It is recognized that human platelets and plasma lipoproteins interact and are intimately involved in the pathogenesis of atherosclerosis, thrombosis, and coronary artery disease.²² In this regard, we report for the first time that HDL, specifically the apoA-I constituent of HDL, helps to maintain the level of platelet SULT2B1b mRNA at 37°C. The physiological meaning of this finding, however, is not presently appreciated and will require additional experimentation to ferret out the significance. Regardless, the fact that platelets lack a nucleus and are thus unable to produce mRNAs suggests that this observation is significant. Could this be a general phenomenon involving all or most platelet mRNAs when these anucleate particles are maintained at 37°C in the absence of HDL? In this regard, the finding that the level of β -actin mRNA seemed to be unaffected at 37°C in the absence of HDL suggests that this may not be a general phenomenon. The rapid degradation of SULT2B1b at 37°C in vitro suggests that such a phenomenon would likely occur in vivo if it were not for the continuous presence of HDL. Thus, an essential role played by HDL involving these anucleate particles is to maintain SULT2B1b mRNA and conceivably other mRNAs at a critical level. Although apoA-I has been shown to bind to platelets,²⁸ the molecular mechanism whereby apoA-I effects stabilization of the mRNA for SULT2B1b at 37°C is not known. Because SULT2B1b mRNA and protein are stable at 4°C, in contrast to 37°C, in the absence of HDL, the involvement of degradative enzymes is suggested.

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